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Brief/Technical Note

Amyloid-Beta Associated with Chitosan Nano-Carrier has Favorable Immunogenicity and Permeates the BBB

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Abstract. Subfragments of amyloid-beta (Aβ) appear to protect neurons from Alzheimer's disease (AD). The permeability of the blood–brain barrier (BBB) has limited *in vivo* research. The aim of this study is to explore permeation of the BBB by chitosan nanoparticles loaded with Aβ and to evaluate immunogenicity of these particles. Chitosan microspheres were prepared by mechanical stirring emulsification methods combined with chemical crosslinking. Morphological characteristics of the nanoparticles were examined using high-resolution transmission electron microscopy. The peptide association efficiency was determined by high-performance liquid chromatography. Fluorescently labeled chitosan nanoparticle-intramembranous fragments of Aβ (NP-IF-A) were administered systemically to mice in order to evaluate brain translocation by fluorescence microscopy. The immunogenicity of the nano-vaccine was determined by enzyme-linked immunosorbent assay (ELISA). All nanoparticles analyzed were well-separated, roughly spherical structures with uniform particle size distribution in the range of 15.23±10.97 nm. The peptide association efficiency was 78.4%. The brain uptake efficiency of nano-antigen was 80.6%; uptake efficiency of antigen alone was only 20.6%. ELISA showed that the nano-vaccine had favorable immunogenicity. A chitosan nano-carrier for Aβ allowed permeation of the BBB. These findings indicate that this novel targeted nano-vaccine delivery system can be used as a carrier for Aβ. This system will further research of peptide vaccines for AD.

KEY WORDS: amyloid-beta; blood–brain barrier; immunogenicity; nanoparticle.

INTRODUCTION

One of the major pathological features of Alzheimer's disease (AD) is the abundance of amyloid plaques in the brains of affected individuals (1). Some subfragments of amyloid-β (Aβ) are protective to neurons and antibodies appear to be cytotoxic (2–5). The blood–brain barrier (BBB) limits the influx of antigens and antibodies to the brain, consequently limiting controlled study of these peptides and antibodies.

Chitosan has important qualities, including low toxicity and muco-adhesive (6) and immunostimulation properties (7). Thus, it has potential to be used as carrier system in research that requires permeation of the BBB. As far as we know, the evaluation of a chitosan-based delivery system for Aβ has not been reported. The main purpose of this work was to evaluate the permeability of the BBB by Aβ (IF-A) associated with chitosan nanoparticles (NP) and to determine the level of immune response produced after intraperitoneal injection (i.p.) into mice.

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MATERIALS AND METHODS

Materials

Chitosan (low molecular weight) was from Aldrich (St. Louis, MO, USA). Glutaraldehyde was from Showa Chemical (Tokyo, Japan). Aβ42, IF-A, fluorescein isothiocyanate in incomplete Freund's adjuvant (FITC-IF-A), and keyhole limpet hemocyanin-IF-A (KLH-IF-A) were synthesized at SciLight Biotechnology, LLC. Freund's adjuvant was from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA), p-nitrophenyl phosphate disodium hexahydrate (PNPP), nitro blue tetrazolium chloride (NBT), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were from Solarbio (Beijing, China). Alkaline phosphatase anti-mouse IgG was from Beijing Dingguo Biotechnology (Beijing, China). All other chemicals used in this study were of analytical grade.

Methods

Preparation of Nanoparticles

Chitosan microspheres were prepared by mechanical stirring emulsification combined with chemical crosslinking according to the procedure described by Jameela *et al.* (8). Briefly, chitosan (1 g) was dissolved in a 1% (w/v) aqueous acetic acid (100 mL) containing 0.9 wt.% sodium chloride. The aqueous phase of this solution (6 g) was dispersed in a

mixture (oil phase: liquid paraffin and petroleum ether in volume ratio of 7:5) of 35 mL of liquid paraffin and 25 mL of petroleum ether containing 0.85 g of sorbitan sesquioleate in a 100-mL round-bottomed flask at room temperature. The dispersion was stirred using a stainless steel half-moon paddle stirrer at 2,000 rev/min for 5 min, and then 1.6 mL of glutaraldehyde was introduced into the flask and the stirring continued. At the end of the 15 min, another 1.6 mL of glutaraldehyde was added. The stirring was continued for a total duration of 1.5 h. The hardened microspheres were then filtered through a fritted disk, washed several times with petroleum ether, washed with methanol, and finally washed with acetone. The microspheres thus obtained were dried overnight in an air oven at 60°C.

Characterization of Nanoparticles by Transmission Electron Microscope and Photon Correlation Spectroscopy

Morphological characteristics of the nanoparticles were examined using high-resolution transmission electron microscope (TEM) (H600, Hitachi, Tokyo, Japan). Freshly made nanoparticles were diluted with deionized water with pH close to neutral. A single drop of sample was syringe placed on a carbon-coated film of 300-mesh copper grid and was air-dried for 10 min. The sample was stained with 1 M uranyl acetate solution for 1.5 min at 7°C and any excess uranyl acetate was removed with filter paper before viewing.

Particle diameters were characterized by photon correlation spectroscopy using a Malvern Zetasizer 3000HS (Malvern Instruments, Westborough, MA, USA) at 258°C with a fixed angle of 908 and a wavelength of 633 nm. The nano-suspensions were diluted with deionized water. All measurements were performed in triplicate.

IF-A Loading

IF-A or FITC-IF-A (0.5 mM) was mixed with 2 mg of chitosan microspheres dissolved in 1 mL of phosphate-buffered saline (PBS) solution. The suspension was kept at 4°C for 48 h under shaking to load IF-A or FITC-IF-A by coupled linkage and adsorption. The antigen-loaded nanoparticles are referred to as nanoparticle-intramembranous fragments of Aβ (NP-IF-A) or NP-FITC-IF-A.

Evaluation of the Peptide Association Efficiency

After a predetermined interval, the IF-A-loaded or FITC-IF-A-loaded chitosan microspheres were separated from the medium by centrifugation at 60,000 r/min for 15 min and the amount of free IF-A in the supernatant was determined by high-performance liquid chromatography (HPLC) (LC26A Jindao, Japan). The mobile phase was a mixture of 0.1% (v/v) TFA/ACN. The column was a Diamohsil TM C18 (250×4.6 mm), the flow rate was 1.0 mL/min, the detection wavelength was 280 nm, and the column temperature was maintained at 30°C. The injected volume of the sample was 20μL. To calculate the association efficiency (AE), we dissolved a known amount of IF-A in PBS and analyzed both free and NP-IF-A by HPLC. The peak area represents the IF-A amount. The AE of the nanoparticle was calculated according to the following equation established by Aktaş *et al.* (9): AE=(total IF-A amount−free IF-A amount)/total IF-A amount×100%.

Animals

Kunming male mice (Department of Animal, Xiangya School of Medicine, Central South University in Changsha, Hunan, China) weighing 18–22 g were used for the experiments. Mice were housed in plastic cages, were maintained on a 12-h light–dark cycle, and had access to food and water *ad libitum*. The animal care was in accordance with institutional guidelines.

Vaccine Administration

Mice were divided randomly into three groups (each with $n=9$): the NP-IF-A group, the KLH-IF-A group, and the control group. NP-IF-A and KLH-IF-A were dissolved in PBS at a concentration of 1 mg/mL (IF-A concentration) and then mixed 1:1 (v/v) with the adjuvant. Complete Freund's adjuvant was used for the first injection, incomplete Freund's adjuvant was used for the next two injections, and the last injection was without adjuvant. Each mouse in the NP-IF-A and KLH-IF-A groups received i.p. injections of 100μg of either NP-IF-A or KLH-IF-A. The first injection was followed by a second injection after 2 weeks, and then mice were dosed monthly. The same volume of PBS replaced antigen for control mice.

Blood was drawn from vena orbitalis posterior on the tenth day following each immunization and serum samples were used to monitor the humoral immune response.

On the tenth day after the last immunization, the mice were anesthetized with diethyl ether. Isotonic sodium chloride (50 mL) was perfused from the left ventricle to clear the blood from the brain. Brains were homogenized in PBS and then the samples were centrifuged at 15,000 r/min for 15 min. Supernatants were evaluated for anti-Aβ IgG.

Enzyme-Linked Immunosorbent Assay for Detection of Anti-Aβ IgG in Serum and Brain

Anti-Aβ IgG titers in serum and brain were quantified by enzyme-linked immunosorbent assay (ELISA). Briefly, microtiter plates were coated overnight at 4°C with 10μg/mL of synthetic Aβ42 in coating buffer (15.1 mM Na_2CO_3, 34.7 mM $NaHCO_3$, pH9). Plates were washed twice with wash buffer (138 mM NaCl, 1.48 mM KH_2PO_4, 2.7 mM KCl, 8.12 mM $Na_2HPO_4{\cdot}12\ H_2O$, 0.82 mM Tween-20) and blocked with 1% BSA for 2 h. Mouse serum and brain homogenate samples were diluted in blocking buffer (1:1,000 v/v) and then were incubated for 2 h with shaking. Plates were washed and then incubated with alkaline phosphatase anti-mouse IgG for 2 h. Plates were washed, incubated with PNPP, and analyzed colorimetrically. The absorbance was determined at 405 nm using a microplate reader.

Brain Uptake of NP-IF-A

Kunming male mice were divided randomly into an experimental group ($n=6$) and the control group ($n=6$). Experimental and control mice were injected with NP-FITC-IF-A and

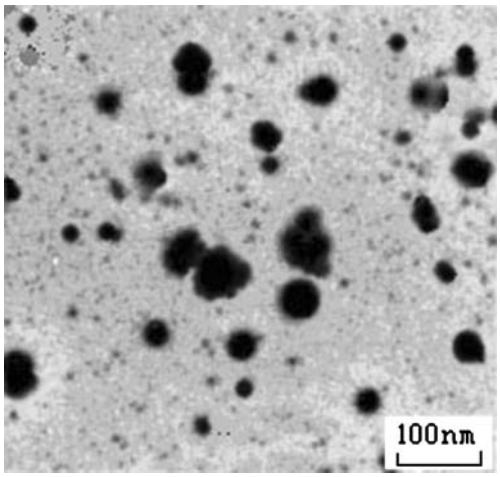

Fig. 1. TEM image of chitosan nanoparticles (NP). Particle size is 15.23±10.97 nm measured by PCS

FITC-IF-A, respectively, into the caudal vein at a dose of 100μg. Mice were anesthetized with diethyl ether after 30 min. Blood was collected from the heart (1 mL), and then 50 mL of isotonic sodium chloride was perfused from the left ventricle to clear the blood from the brain. Brains were homogenized in PBS and then centrifuged at 15,000 r/min for 15 min. Serum and supernatant were analyzed for FITC-IF-A using fluorescence spectrophotometry and microscopy. Brain uptake efficiency was determined as follows: efficiency=OD in brain/OD in blood×100%.

Statistical Analysis

Data were expressed as the mean±standard deviation (SD). Differences were evaluated for significance using an analysis of a two-sample/group *t* test. A two-sided probability level of 0.05 was defined as statistically significant. All calculations were made using statistical software (SPSS 11.0).

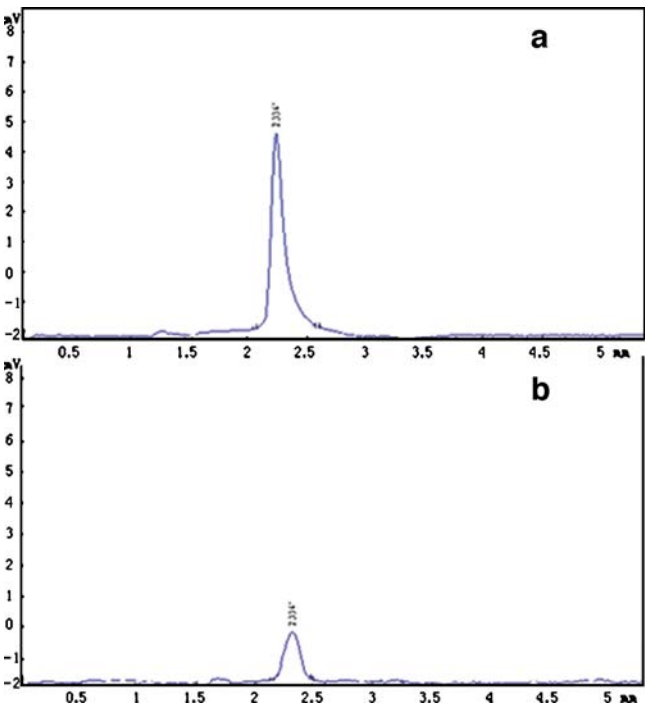

Fig. 2. IF-A association efficiency with NP evaluated by HPLC. The AE is 78.4%. **a** represents the total amount of IF-A, **b** represents the free amount of IF-A

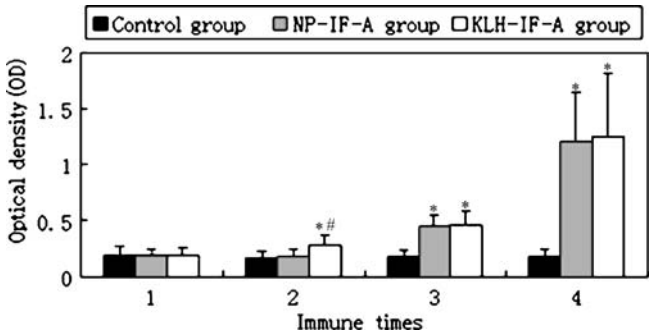

Fig. 3. Plasma IgG anti-A determined by ELISA after four times immunization with NP-IF-A and KLH-IF-A as antigen respectively. There was favourable increase in IgG against Aβ42 in serum recognizing the antigen, especially after the fourth immunization, and there was not significant difference in immunogenicity between NP-IF-A and KLH-IF-A. Data are presented as mean±S.D. *$p<0.05$ versus control group; #$p<0.05$ versus NP-IF-A- group

RESULTS

Characterization of Nanoparticles by TEM and Photon Correlation Spectroscopy

All nanoparticles analyzed by TEM were well-separated, roughly spherical structures with uniform particle size distribution as shown in Fig. 1. The average diameter was 15.23± 10.97 nm as measured by photon correlation spectroscopy (PCS).

AE of Nanoparticles Loaded with IF-A

The AE for IF-A with the nanoparticles was determined as shown in Fig. 2. The AE was 78.4%, favorable for a vaccine.

Serum and Brain IgG Against Aβ42

We evaluated the immunogenicity of NP-IF-A and KLH-IF-A by ELISA. Animals immunized with NP-IF-A and

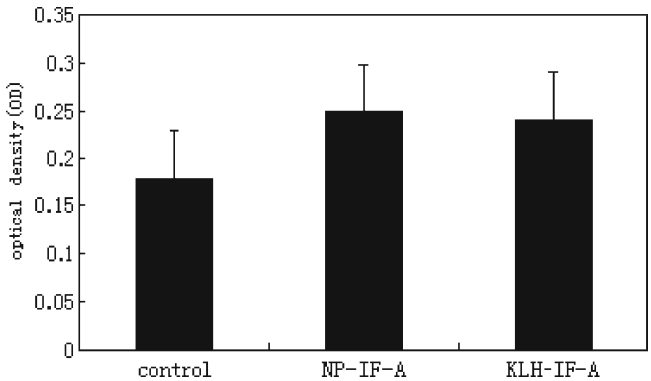

Fig. 4. Brain IgG anti-A antibody determined by ELISA after the last immunization, but the result did not has significant difference between the two antigen (NP-IF-A and KLH-IF-A)

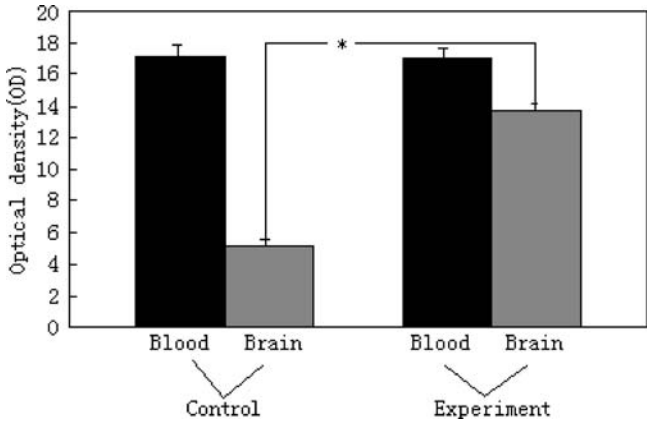

Fig. 5. Evaluation of brain uptake efficiency of the NP-IF-A by fluorescent spectrophotometer. The uptake efficiency of IF-A in experiment group and control group was 80.6% and 20.7% respectively. There was significant difference in brain uptake efficiency between NP-FITC-IF-A and FITC-IF-A, $*p<0.01$

KLH-IF-A had significantly more IgG against Aβ42 in serum than controls, especially after the fourth immunization (Fig. 3). Although the IgG titer against Aβ42 in animals immunized with NP-IF-A was higher than that of those immunized with KLH-IF-A, the result was not a statistically significant difference. There was a lower titer of IgG against Aβ42 in the brain (Fig. 4) than in plasma after the last immunization.

In vivo Brain Uptake of the NP-IF-A

We labeled IF-A with FITC and evaluated the brain uptake efficiency of the NP-IF-A. We compared the IF-A amount in the brain with that in blood and the IF-A amount in the brain in the experimental group with that in the control group by fluorescence spectrophotometry and fluorescent microscopy. The uptake efficiencies of IF-A in the experimental group and the control group were 80.6% and 20.7%, respectively. There was a significant difference between the two groups, evaluated by fluorescent spectrophotometer ($p<0.01$) (Figs. 5 and 6).

DISCUSSION

Chitosan has been used as a safe excipient in drug formulations for over two decades (10). Its lack of toxicity and allergenicity and its biocompatibility, biodegradability, and bioactivity make it a very attractive substance for diverse applications in pharmaceutical and medical fields. It has been used for both systemic and local delivery of drugs and vaccines (11,12).

Glutaraldehyde is somewhat toxic, but glutaraldehyde-crosslinked chitosan microspheres have been extensively investigated for drug delivery (13–15), and different crosslinking methods (i.e., sulphuric acid and heat treatment) should be explored. Different preparation methods, types of chitosan, and different crosslinking methods result in nanoparticle size differences (16). Particle size measurements depend on the detection method (17). The discrepancy in the sizes of nanoparticles based on PCS and TEM may be due to the fact that the dynamic light scattering method gives the hydrodynamic diameter rather than the actual diameter of nanoparticles. A comparison of the particle sizes determined using other techniques would be valuable.

Aβ vaccine can promote humoral immunity and production of antibodies against Aβ, reducing the Aβ burden and

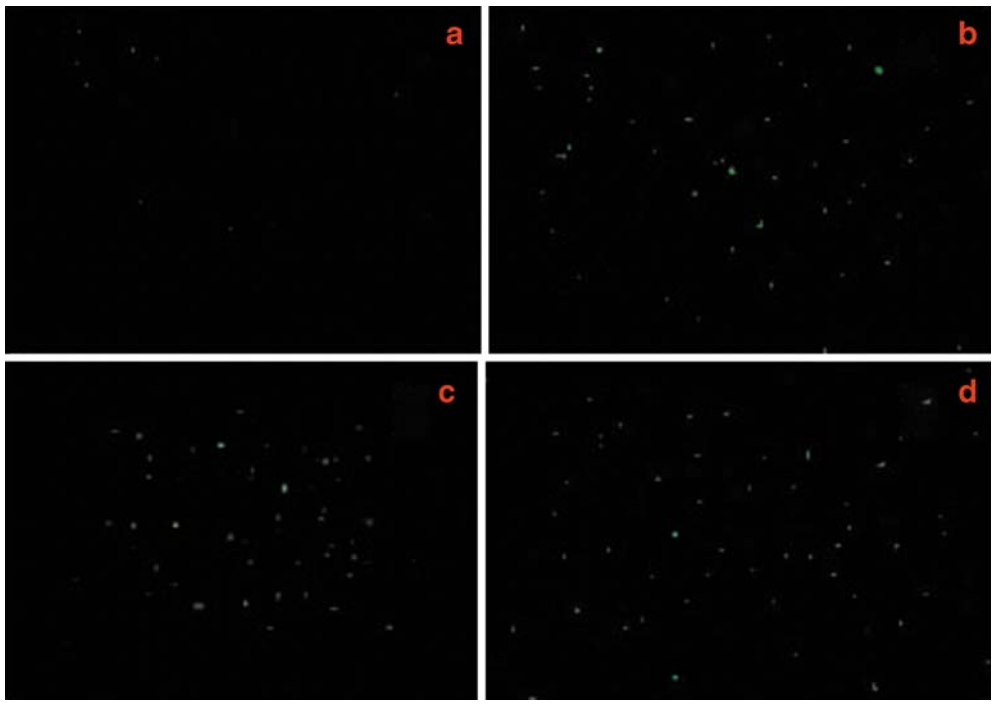

Fig. 6. Evaluation of brain uptake efficiency of NP-IF-A with fluorescent microscope. "**a**" and "**b**" represents FITC-IF-A in brain and blood respectively in control group; "**c**" and "**d**" represents NP-FITC-IF-A in brain and blood respectively in experimental group

behavioral impairment in AD models (18,19). Several researchers have reported that chitosan has the ability to regulate the functions of cellular immunity (20–22) and macrophages (23,24). However, there have been no analyses of the effect of chitosan on humoral immunity. In the present study, we injected IF-A linked to chitosan nanoparticles into Kunming mice. The IF-A-NPs elicited favorable titers of anti-amyloid antibodies in plasma and in the brain, though the increased titer of antibody in the brain was not significantly different compared with that of the KLH-IF-A vaccine. It will be necessary to further explore the potential of chitosan in this immune reaction.

The BBB is a diffusion barrier essential for the normal function of the central nervous system. Endothelial cell tight junctions limit the paracellular flux of hydrophilic molecules across the BBB (25). Consequently, very few molecules are transported efficiently into the brain, which compromises the efficient treatment of neurological and psychiatric disorders. In the swollen state, chitosan adheres to epithelial tissues and to the mucus coating presenting on the surface of the tissues. In this study, the chitosan nano-vaccine carrier had favorable permeability of BBB with FITC as a marker. The covalent bond between the isothiocyano group in FITC and amino groups on Aβ (mainly the lysine epsilon amino group) is very stable *in vivo*. It is reasonable that the light intensity of FITC represents the amount of Aβ present in tissue. Aktas *et al*. (9) used fluorescence microscopy to detect brain uptake of FITC-labeled NPs and confirmed that this novel targeted nanoparticle drug delivery system was able to translocate into the brain tissue after intravenous administration. We are currently further exploring the mechanism of its high BBB permeability.

CONCLUSIONS

A new type of chitosan nanoparticle was synthesized as an Aβ carrier using mechanical stirring emulsification methods to achieve a high linkage efficiency to Aβ antigen. The nanoparticles demonstrated efficient permeability of BBB and significant immunogenicity was observed. These findings indicate that this novel nano-delivery system can be used to carry Aβ to the brain. This system will further research of peptide vaccines for AD.

ACKNOWLEDGMENTS

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